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(54) Title: STEM CELL CULTURE

(57) Abstract: We describe a method to manipulate the phenotype of stem cells, preferably embryonic stem cells (ES), including nucleic acids and vectors used in said methods.

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Stem Cell Culture

The invention relates to a method to manipulate the phenotype of stem cells, preferably embryonic stem cells (ES), including nucleic acids and vectors used in said methods.

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During mammalian development those cells that form part of the embryo up until the formation of the blastocyst are said to be totipotent (e.g. each cell has the developmental potential to form a complete embryo and all the cells required to support the growth and development of said embryo). During the formation of the blastocyst, the cells that comprise the inner cell mass are said to be pluripotent (e.g. each cell has the developmental potential to form a variety of tissues).

10 Embryonic stem cells (ES cells, those with pluripotentiality) may be principally derived from two embryonic sources. Cells isolated from the inner cell mass are termed embryonic stem (ES) cells. In the laboratory mouse, similar cells can be derived from the culture of primordial germ cells isolated from the mesenteries or genital ridges of days 15 8.5-12.5 *post coitum* embryos. These would ultimately differentiate into germ cells and are referred to as embryonic germ cells (EG cells). Each of these types of pluripotential cell has a similar developmental potential with respect to differentiation into alternate cell 20 types, but possible differences in behaviour (eg with respect to imprinting) have led to these cells to be distinguished from one another.

25 Typically ES/EG cell cultures have well defined characteristics. These include, but are not limited to; maintenance in culture for at least 20 passages when maintained on fibroblast feeder layers; produce clusters of cells in culture referred to as embryoid bodies; ability to differentiate into multiple cell types in monolayer culture; and express ES/EG cell specific markers.

30 Until very recently, *in vitro* culture of human ES/EG cells was not possible. The first indication that conditions may be determined which could allow the establishment of human ES/EG cells in culture is described in WO96/22362. The application describes

cell lines and growth conditions which allow the continuous proliferation of primate ES cells which exhibit a range of characteristics or markers which are associated with stem cells having pluripotent characteristics.

5 More recently Thomson *et al.* (1998) have published conditions in which human ES cells can be established in culture. The above characteristics shown by primate ES cells are also shown by the human ES cell lines. In addition the human cell lines show high levels of telomerase activity, a characteristic of cells which have the ability to divide continuously in culture in an undifferentiated state. Another group (Reubinoff *et. al.*,
10 2000) have also reported the derivation of human ES cells from human blastocysts. Shambrott *et. al.*, 1998 have also described EG cell derivation. In Lake *et al* J Cell Science 2000, 113:555-66 and Rathjen et al J Cell Science 1999, 112: 601-12, ectodermal stem cells are disclosed. The above references are each both incorporated by reference in their entirety.

15 A problem associated with the maintenance of ES/EG cells in culture is that it is extremely difficult to maintain a pure population of proliferating ES/EG cells. ES/EG cells require the presence of fibroblast feeder layers in order to retain their ability to divide in an undifferentiated state for several generations. If the feeder layers are removed then the cells differentiate. The differentiation is often to neurones or muscle cells but the exact mechanism by which this occurs and its control remains unsolved. A further problem is that even when grown under optimal conditions, the cells tend to spontaneously differentiate leading to cultures in which undifferentiated cells are admixed with variable numbers of their differentiated derivatives.

20 25 Fibroblast growth factor-4 (FGF-4), also referred to as heparin secretory transforming protein 1 (HST-1) or Kaposi sarcoma oncogene, is a member of the fibroblast growth factor family. FGF-4 has been shown to be a signalling molecule involved in growth and differentiation via the dimerisation of its tyrosine kinase receptors. *In vivo*, the FGF-4 gene is expressed in the blastocyst inner cell mass and in specific embryonic tissues, but is transcriptionally silent in the adult (Yuan *et al.*, 1995). Likewise, in culture, FGF-4

expression has also been shown to be restricted to undifferentiated embryonic stem cells and embryonal carcinoma (EC) cell lines (Yuan *et al.*, 1995). Expression of FGF-4 is controlled by cis-regulatory elements located in the 5'-flanking region and by a powerful enhancer located approximately 3 kb downstream from the transcription start site (Lamb *et al.*, 1996). Repression of FGF-4 gene transcription occurs in differentiated cells due to a reduction in the ability of nuclear factors to bind to an octamer motif and an adjacent High Mobility Group motif within the enhancer.

10 *Rex-1* (*Zfp-42* gene) is a developmentally regulated acidic zinc finger gene. *Rex-1* mRNA demonstrates a similar expression profile to FGF-4 and is detected in undifferentiated embryonic stem and EC cells, mouse embryos at the blastocyst stage, and also in the trophectoderm and meiotic germ cells of the adult mouse testis (Rogers *et al.*, 1991). Transcription of *Rex-1* is reduced during retinoic acid-induced differentiation (Hosler *et al.*, 1993). An octamer motif (ATTTGCAT) at position -220 in the murine *Rex-1* promoter has been shown to be required for promoter activity in mouse F9 teratocarcinoma cells (Hosler *et al.*, 1993). This octamer motif is a binding site for members of the POU (Pit-Oct-Unc) domain family of DNA-binding proteins (Anderson & Rosenfeld, 2001), including Oct-3/4 and its alternatively spliced forms Oct-5 and-6, all expressed by EC and ES cells (Schöler 1991). Oct-3 has been shown to bind to the 15 ATGCAAAT motif in *Rex-1* (Ben-Shushan *et al.*, 1998) and FGF-4 (Ambrosetti *et al.*, 1997).

20 Selectable markers can be used to select a required genotype. These markers confer a new phenotype, often based on resistance to antibiotics such as ampicillin, (Sykes & Matthew, 1976) tetracycline (Franklin, 1967), chloramphenicol (Le Grice & Matzura, 1981), kanamycin (neomycin), hygromycin and puromycin (Karreman *et al.*, 1998). However, the drug concentrations required to reduce the survival of cells carrying "unwanted" 25 genotypes may have a long-term effect on the cells, for example, levels have been shown to inhibit progenitor cell proliferation and/or differentiation in hematopoietic stem cells (Chinswangwatanakul *et al.*, 1998). An alternative strategy for selection of living cells is 30 based on the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, and

variants thereof, including blue-(BFP), cyan-(CFP) and yellow(YFP)- fluorescent protein (Beavis *et al.*, 1999) and also red fluorescent protein (RFP) from *Discosoma* coral (Matz *et al.*, 1999).

5 Suicide gene therapy is one of several gene therapeutic approaches to treat disease, particularly cancer. A suicide gene is a gene, usually of viral or prokaryotic origin that encodes a protein, typically an enzyme, that in itself is non-toxic to the genetically modified cell. However, when a cell is exposed to a specific non-toxic prodrug, this is selectively converted by the gene product into toxic metabolites that kill the cell (See
10 Table 1). The suicide gene most commonly employed, both in experimental and clinical settings, is herpes simplex thymidine kinase (HSVtk). For example a c-erb-2 promoter-mediated expression of HSVtk has been shown to confer selective cytotoxicity of human breast cancer cells to gancyclovir (Maeda *et al.*, 2001). Other examples of suicide genes are those which express products that are directly toxic to the cell, such as the diphteria
15 toxin or pseudomonas exotoxin or which inhibit protein synthesis (Dilber & Gahrton, 2001).

We have now examined expression of FGF4 in stock cultures of human ES cells. If these cells are sorted for expression of the surface antigen marker SSEA3 characteristically
20 expressed by undifferentiated human ES cells, (Thomson *et al* 1998; Draper *et al* 2002), we have found expression of FGF4 exclusively in the SSEA3-positive cells, and not in the SSEA3-negative cells that constitute their spontaneous differentiated derivatives (Figure 1). By contrast, the SSEA3-negative cells expressed predominantly or exclusively other marker genes characteristic of differentiated derivatives – e.g. AFP and
25 HBZ (yolk sac and embryonic hematopoietic cells), HCG (trophectoderm), ND1, Sox1 (neural cells).

We have determined that the inclusion of a motif from the FGF4 enhancer in a promoter, for example, the thymidine kinase promoter, confers stem cell specific expression on
30 reporter genes under its control. We have exploited this discovery to provide a cell culture system which facilitates the maintenance of stem cells, particularly embryonic

stem cells, in an undifferentiated state. We also provide differentiated cells/tissues the genome of which includes a nucleic acid construct comprising a promoter which has substantially a stem cell specific expression pattern which controls expression of a gene the expression of which allows the selective ablation of cells which have de-differentiated 5 to a stem cell phenotype thereby allowing their removal from a population of differentiated cells. The invention therefore relates, amongst other things, to the provision of methods to manipulate the phenotype of stem cells, preferably embryonic stem cells (ES).

10 According to an aspect of the invention there is provided a method to manipulate stem cell phenotype comprising;

- (i) providing a stem cell which has been transfected with a nucleic acid molecule wherein said molecule includes a promoter which comprises at least one nucleic acid sequence motif which confers substantially stem cell specific expression on 15 at least one selectable marker gene;
- (ii) providing conditions conducive to the proliferation of said cell in (i) above; and optionally
- (iii) maintaining and/or storing said cell.

20 In a preferred method of the invention said conditions are *in vitro* cell culture conditions.

In a further preferred method of the invention said promoter is a synthetic promoter. A synthetic promoter maybe a minimal promoter which includes motifs which direct stem 25 cell specific expression with a core promoter (e.g. heatshock promoters HSP68, thymidine kinase promoter).

In a preferred method of the invention, the motif binds the transcription factors of the POU domain family, more specifically Oct-3/4 and its alternatively spliced forms Oct-5 30 and-6.

In a further preferred method of the invention, the motif is derived from the FGF-4 promoter, preferably of human origin. Preferably said motif is at least one copy of the sequence CTTTGTT and ATGCAAAT ; or CTTTGTT and ATGCTAAT.

5 In a preferred method of the invention said motif comprises at least one copy of the motif CTTTGTTXATGCAAAT wherein X is at least one nucleotide base which serves as a linking molecule.

10 In an alternative method of the invention said motif comprises at least one copy of the motif CTTTGTTXATGCTAAT wherein X is at least one nucleotide base which serves as a linking molecule.

An example of such a motif is CTTTGTTcgaATGCAAAT.

15 In a further preferred method of the invention said nucleic acid molecule comprises a plurality of motifs according to the invention. Preferably there is provided at least 2 motifs. More preferably still there is provided at least 3, 4, 5, 6, 7, 8, 9 or 10 motifs.

20 In a further preferred method of the invention the motif is derived from the *Rex-1* (*zfp-42*) promoter, preferably *Rex-1* is of human origin.

In an alternative preferred method of the invention the motif is derived from the murine *Rex-1* promoter, for example, ATTTGCAT.

25 In a further preferred method of the invention stem cells are selected from a group consisting: haemopoietic stem cells; neural stem cells; bone stem cells; muscle stem cells; mesenchymal stem cells; trophoblastic stem cells; epithelial stem cells (derived from organs such as the skin, gastrointestinal mucosa, kidney, bladder, mammary glands, uterus, prostate and endocrine glands such as the pituitary); endodermal stem cells
30 (derived from organs such as the liver, pancreas, lung and blood vessels); embryonic stem

(ES) cells; embryonal germ (EG) cells. Preferably said stem cells are primate, preferably human.

In an alternative further preferred method of the invention said stem cells are embryonal carcinoma cells. Preferably said embryonal carcinoma cells are TERA2 cells. Ideally said embryonal carcinoma cells are NTERA 2 cells.

5 In a yet further preferred method of the invention said selectable marker is a gene encoding a polypeptide capable of fluorescence emission when excited by light. More 10 preferably still said polypeptide is selected from the group consisting of; BFP, CFP, YFP, GFP, RFP and variants thereof.

GFP of the jelly fish *Aequorea victoria* has an excitation maximum 395nm, an emission at 510nm and does not require the addition of an exogenous factor. Mutant forms of GFP 15 are also known with altered fluorescence emission properties, see WO9821355; US5804387; US5777079; and US5625048, which are incorporated by reference.

In an alternative preferred method of the invention said selectable marker is a gene 20 encoding a polypeptide which confers resistance to antibiotics selected from the group consisting of; β lactamase, tetracycline resistance polypeptide, chloramphenicol acetyltransferase, aminoglycoside phosphotransferase, hygromycin phosphotransferase; puromycin N acetyltransferase, or variants thereof.

According to a further aspect of the invention there is provided an isolated nucleic acid 25 molecule comprising a promoter of a gene which has substantially stem cell specific expression operably linked to at least one selectable marker.

In a preferred embodiment of the invention said promoter comprises a motif which 30 confers substantially stem cell expression of said selectable marker. Preferably said motif is bound by a transcription factor of the POU family of transcription factors, for example Oct-3/4 and its alternatively spliced forms Oct-5 and-6.

Preferably said motif is at least one copy of the sequence CTTTGTT and ATGCAAAT ; or CTTTGTT and ATGCTAAT.

5 In a preferred method of the invention said motif comprises at least one copy of the motif CTTTGTTXATGCAAAT wherein X is at least one nucleotide base which serves as a linking molecule.

10 In an alternative method of the invention said motif comprises at least one copy of the motif CTTTGTTXATGCTAAT wherein X is at least one nucleotide base which serves as a linking molecule.

In an alternative preferred embodiment said motif is derived from the *Rex-1 (zfp-42)* promoter, preferably human *Rex-1 (zfp-42)* or murine *Rex-1*.

15 In a further preferred embodiment of the invention said selectable marker encodes a prodrug activating polypeptide.

20 In a preferred embodiment of the invention said prodrug activating polypeptide is selected from the group consisting of: thymidine kinase; triphosphate cytosine deaminase; 5' guanosine -xanthine phosphoribosyl transferase ; purine nucleoside phosphorylase; nitroreductase; CYP 2B1 (cytochrome P450); CYP 4B1 (cytochrome P450); Cytochrome P450; Varicella zoster virus thymidinekinase (VZVtk); β -glucosidase; β -lactamase; β -glucuronidase; Carboxylesterase; Alkaline phosphatase; 25 carboxypeptidase G2, or variants thereof.

According to a further aspect of the invention there is provided a vector comprising a nucleic acid molecule according to the invention.

30 Typically the vector is viral based. Preferred vectors are derived from the adenoviral, adeno-associated viral or retroviral genomes. The vectors can be derived from the

human adenovirus genome. Particularly preferred vectors are derived from the human adenovirus serotypes 2 or 5. The replicative capacity of such vectors may be attenuated (to the point of being considered "replication deficient") by modifications or deletions in the E1a and/or E1b coding regions. Other modifications to the viral genome to achieve 5 particular expression characteristics or permit repeat administration or lower immune response are preferred. Most preferred are human adenoviral type 5.

Alternatively, the viral vectors may be conditionally replicating or replication competent. Conditionally replicating viral vectors are used to achieve selective expression in 10 particular cell types while avoiding untoward broad spectrum infection. Examples of conditionally replicating vectors are described in Pennisi, E. (1996) *Science* 274:342-343; Russell, and S.J. (1994) *Eur. J. of Cancer* 30A(8):1165-1171. Additional examples of selectively replicating vectors include those vectors wherein an gene essential for replication of the virus is under control of a promoter which is active only in a particular 15 cell type or cell state such that in the absence of expression of such gene, the virus will not replicate. Examples of such vectors are described in Henderson, et al., United States Patent No. 5,698,443 issued December 16, 1997 and Henderson, et al., United States Patent No. 5,871,726 issued February 16, 1999 the entire teachings of which are herein incorporated by reference.

20 The viruses may also be designed to be selectively replicating viruses. Particularly preferred selectively replicating viruses are described in Ramachandra, et al. PCT International Publication No. WO 00/22137, International Application No. PCT/US99/21452 published April 20, 2000 and Howe, J., PCT International Publication No. WO 00/22136, International Application No. PCT/US99/21451 published April 20, 25 2000.

It has been demonstrated that viruses which are attenuated for replication are also useful in the therapeutic arena. For example the adenovirus dl1520 containing a specific deletion in the E1b55K gene (Barker and Berk (1987) *Virology* 156: 107) has been used with therapeutic effect in human beings. Such vectors are also described in McCormick

(United States Patent No. 5,677,178 issued October 14, 1997) and McCormick, United States Patent No 5,846,945 issued December 8, 1998.

According to a further aspect of the invention there is provided a stem cell transfected
5 with a DNA molecule or a vector according to the invention.

In a preferred embodiment of the invention said cell is stably transfected. Alternatively
said cell is transiently transfected.

10 In a preferred embodiment of the invention said stem cell is selected from the group
consisting of: haemopoietic stem cells; neural stem cells; bone stem cells; muscle stem
cells; mesenchymal stem cells; trophoblastic stem cells; epithelial stem cells (derived
from organs such as the skin, gastrointestinal mucosa, kidney, bladder, mammary glands,
uterus, prostate and endocrine glands such as the pituitary); endodermal stem cells
15 (derived from organs such as the liver, pancreas, lung and blood vessels).

Preferably said cell is an embryonic stem cell. Alternatively said cell is an embryonal
carcinoma cell. Preferably said embryonal carcinoma cells are TERA2 cells. Ideally said
embryonal carcinoma cells are NTERA 2 cells.

20 According to a further aspect of the invention there is provided a differentiated cell
transfected with a DNA molecule or vector according to the invention.

25 In a preferred embodiment of the invention said differentiated cell is selected from the
group consisting of: a nerve cell; a mesenchymal cell; a muscle cell (cardiomyocyte); a
liver cell; a kidney cell; a blood cell (eg erythrocyte, CD4+ lymphocyte, CD8+
lymphocyte; panceatic β cell; epithelial cell (eg lung, gastric,); and a endothelial cell.
30 Conventional methods to introduce DNA into cells are well known in the art and
typically involve the use of chemical reagents, cationic lipids or physical methods.

Chemical methods which facilitate the uptake of DNA by cells include the use of DEAE-Dextran (Vaheri and Pagano Science 175: p434) . DEAE-dextran is a negatively charged cation which associates and introduces the DNA into cells but which can result in loss of cell viability. Calcium phosphate is also a commonly used chemical agent which when co-precipitated with DNA introduces the DNA into cells (Graham et al 5 Virology (1973) 52: p456).

The use of cationic lipids (eg liposomes (Felgner (1987) Proc.Natl.Acad.Sci USA, 84:p7413) has become a common method since it does not have the degree of toxicity 10 shown by the above described chemical methods. The cationic head of the lipid associates with the negatively charged nucleic acid backbone of the DNA to be introduced. The lipid/DNA complex associates with the cell membrane and fuses with the cell to introduce the associated DNA into the cell. Liposome mediated DNA transfer has several advantages over existing methods. For example, cells which are recalcitrant to 15 traditional chemical methods are more easily transfected using liposome mediated transfer.

More recently, other non-liposome based chemical transfectant agents have become 20 available, for example ExGen500 (polyethylenimine), produced by MBI Fermentas. ExGen500 is particularly effective for transfection of human ES cells (Eiges, 2001).

More recently still, physical methods to introduce DNA have become effective means to 25 reproducibly transfet cells. Direct microinjection is one such method which can deliver DNA directly to the nucleus of a cell (Capecchi (1980) Cell, 22:p479). This allows the analysis of single cell transfectants. So called "biolistic" methods physically shoot DNA into cells and/or organelles using a particle gun (Neumann (1982) EMBO J, 1: p841). Electroporation is arguably the most popular method to transfet DNA. The method involves the use of a high voltage electrical charge to momentarily permeabilise cell membranes making them permeable to macromolecular complexes. However physical 30 methods to introduce DNA do result in considerable loss of cell viability due to

intracellular damage. These methods therefore require extensive optimisation and also require expensive equipment.

More recently still a method termed immunoporation has become a recognised technique for the introduction of nucleic acid into cells, see Bildirici *et al* Nature (2000) 405, p298. The technique involves the use of beads coated with an antibody to a specific receptor. The transfection mixture includes nucleic acid, typically vector DNA, antibody coated beads and cells expressing a specific cell surface receptor. The coated beads bind the cell surface receptor and when a shear force is applied to the cells the beads are stripped from the cell surface. During bead removal a transient hole is created through which nucleic acid and/or other biological molecules can enter. Transfection efficiency of between 40-50% is achievable depending on the nucleic acid used. The above described methods for the introduction of DNA into cells are incorporated by reference.

15 According to a further aspect of the invention there is provided a cell culture comprising a transfected stem cell according to the invention.

According to a yet further aspect of the invention there is provided a cell culture comprising a differentiated cell according to the invention.

20 According to a yet further aspect of the invention there is provided at least one organ/tissue comprising at least one differentiated cell according to the invention.

25 According to a yet further aspect of the invention there is provided a method to ablate a cell according to the invention comprising exposing said cell to an agent to which said cell has been sensitized.

In a preferred method of the invention said cell is a differentiated cell which has or is de-differentiating to a stem cell or lineage restricted stem cell.

30 In a further preferred method of the invention said method is an *in vivo* method.

In an alternative preferred method of the invention said method is an *in vitro* method.

In a further preferred method said agent is selected from the group consisting of:

5 ganciclovir; 5-fluorouracil (5-FU); 6-Tg triphosphate; 6-methylpurine; hydroxylamine; 4-hydroperoxycyclo-phosphoamide; DNA-alkylating; alkylating metabolites; araM-MP; cyanide; vinca alk; phenolmustard; SN-38; phenolmustard; etoposide; benzoic acid mustards.

10 In an alternative preferred method of the invention said agent is diphtheria toxin or pseudomonas exotoxin.

An embodiment of the invention will now be described by example only and with reference to the following table and figures:

15 Table 1 represents a summary of prodrug activating enzymes and their respective prodrugs and activated drugs;

Table 2 illustrates the PCR primers used to detect gene expression in human ES cells;

20 Figure 1 is a schematic diagram of FGF4 promoter cassettes illustrating DNA binding motifs; Human/Mouse Sox2 DNA binding motif: CTTTGTT Human Oct4 DNA binding motif: ATGCAAAT Mouse Oct4 DNA binding motif: ATGCTAAT;

25 Figure 2 represents the nucleic acid sequences of minimal human and murine FGF4 promoters;

30 Figure 3 shows reverse transcriptase (RT)-PCR analysis of SSEA3(+) and (-), and SSEA1(+) and (-), subsets of H7 human ES cells isolated using fluorescence activated cell sorting. After screening for genomic contamination (data not shown), 1 μ g total RNA was reverse transcribed into first-strand cDNA, which was then subject to PCR using

primers specific to human Oct4, Sox2, FGF4, Rex1, AFP, HBZ, HCG, NeuroD1 (ND-1), Sox1 and β Actin (loading control). Note that especially FGF4 is exclusively expressed in the SSEA3 positive cells which correspond to the undifferentiated cells (Draper et al 2002). By contrast genes typical of differentiated derivatives (e.g. α FP, HB2, HCG, 5 ND-1, SOX1) are exclusively or predominately expressed in the SSEA-3 negative cells;

Figure 4 illustrates examples of GFP expression vector constructs;

Figure 5 illustrates the sequence elements of a GFP expression vector construct;

10 Figure 6 illustrates transfection of EC/ES cells with GFP expression vectors including FGF4 enhancer motifs. Human EC cell line NTERA2/D1 was transfected with a control plasmid (pTK-GFP) containing only the TK basal promoter and GFP (Fig 4 A and B – fluorescence and phase respectively). Note the low level basal GFP expression provided by the TK promoter. p5FGF4EN-GFP, a plasmid with four copies of the human Sox2/Oct4 binding motif inserted immediately 5' to the TK promoter of pTK-GFP, was 15 also transfected into NTERA2/d1 (Fig 4 C and D). Note the substantially enhanced GFP expression compared to pTK-GFP alone. Similar results were obtained with H7 hES cells transfected with the same pair of vectors;

20 Figure 7 is a FACS analysis of EC cells transfected with a FGF 4 reporter plasmid. Two fluorochrome flow cytometric analysis using p5FGF4EN-GFPIP reporter combined with SSEA-3 labelling of undifferentiated NTERA2 human EC cells (A) and retinoic acid treated cells (B) demonstrates that GFP expression from FGF-4 reporter is greatly 25 reduced upon differentiation; this reduction is concomittant with a reduction in SSEA-3 expression. The same experiment performed using the pTK-GFPIP control vector which lacks the 4x Oct4/Sox-2 binding motifs is included for comparison; and

30 Figure 8 is a FACS analysis of EC and ES cells transfected with various reporter constructs. Transient transfection of human EC cells (2102EP; A) or ES cells (H7;B) with an FGF-4 enhancer reporter (p5FGF4EN) show that there is a substantial increase in

fluorescence when compared to an identical vector lacking the Sox/Oct 4 binding motifs (pTK-GFPPIP). Chi squared based statistical analysis of co-labelling of transfected cells with surface markers characteristic of an undifferentiated phenotype (SSEA-3, TRA-1-60) or differentiated derivatives (SSEA-1) indicates that FGF-4 enhancer activity is 5 associated with, for example, TRA-1-60 in human ES cells.

Materials and Methods

GFP Vector Constructs

10 Three mammalian expression vectors encoding GFP were constructed

1. pTK-GFP: The expression of GFP was driven only by the minimal promoter from the HSV-Tk gene (TK minimal promoter was inserted into the AgeI/Apal restriction sites in pd2EGFP-1 (clontech)).
- 15 2. p5FGF4EN-GFP: The expression of GFP was driven by an enhancer construct incorporating 4 copies of the human FGF4 enhancer inserted 5' to the HSV-Tk minimal promoter (4x human Sox2/Oct4 DNA binding motif was inserted in the BgIII/EcoRI restriction sites immediately 5' to the TK promoter of pTK-GFP).

20 p5FGF4EN-Bact-GFP. This construct is similar to p5FGF4EN-GFP, except that two pairs of 2x human Sox2/Oct4 DNA binding motifs are separated by a 400bp region of β -actin.

25 Variations of these vectors including pTK-GFP, p5FGF4EN-GFP and p5FGF4EN-Bact-GFP with IRES site linking an antibiotic selection marker, for example puromycin or neomycin resistance, to the expression of GFP were also created (IRES-antibiotic selection marker sequences were inserted in the NotI cloning site immediately 3' to the GFP sequence).

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Cell Lines and Culture Conditions

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The human embryonal carcinoma (EC) cell line, NTERA2 cl D1, was cultured in DMEM with 10% fetal calf serum as previously described (Andrews et al 1984). Cells were harvested using 0.25% trypsin and 1 mM EDTA in calcium and magnesium free Dulbecco's phosphate buffered saline as previously described (Andrews 1984) and 10 seeded at 6×10^4 cells per cm^2 the day before transfection.

The hES cell line H7 and H14 (Thomson et al 1998) were cultured in "Knock-Out" DMEM (GICO Life Technologies Ltd) supplemented with 20% Serum Replacement (GIBCO Life Technologies Ltd) at 37 C under a humidified atmosphere of 5% CO_2 in 15 air, on mitomycin C inactivated mouse embryo fibroblasts (Thomson et al 1998). The cells were harvested by exposure for 3 min at 37 C to a solution of 0.05% trypsin in Calcium and Magnesium free Dulbecco's Phosphate Buffered saline containing 1 mM EDTA. When the cells detached they were resuspended in fresh medium and plated into a fresh tissue culture vessel, precoated with a 1:30dilution of Matrigel (Becton 20 Dickinson), at 6×10^4 cells per cm^2 .

Alternatively, for subcultivation, the cells were harvested by treatment with 1 mg/ml collagenase IV, and dispersed by scraping, to maintain the cells in small clumps.

Differentiation was induced by incorporating all-*trans*-retinoic acid (Eastman-Kodak) 25 (10^{-5} M) into the medium as described for human EC cells (Andrews, 1984).

Cell Transfection

Human EC and ES cells were transfected by the same protocol: After incubation at 37° C 30 overnight the cells were transfected, following the manufacturer's protocol, as follows: 9.5 μg DNA was diluted into 300 μl 0.15 M NaCl and mixed with 21 μl ExGen 500 (MBI Fermentas) and vortexed. After incubating at room temperature for 10 min, the DNA:

ExGen solution was mixed with 3 ml culture medium and added a culture of ES cells as described above in a 35 mm (9.6 cm²) dish. After incubating for 6 – 12 hours at 37 C, the medium was removed and the cells fed with fresh medium and fluorescence observed by microscopy under UV epi-illumination.

5 **Surface Antigen Expression**

Cell surface antigen expression of cultured cells was assessed by indirect immunofluorescence detected by flow cytofluorimetry, after harvesting the cultures as single cell suspensions using trypsin-EDTA, as described previously (Andrews *et al* 1987, Fenderson *et al* 1987). The following primary monoclonal antibodies were used to 10 detect surface antigen expression: MC631, anti stage specific embryonic antigen 3 (SSEA3) (Shevinsky *et al* 1982), MC813-70, anti stage specific embryonic antigen 4 (SSEA4) (Kannagi *et al* 1983), MC480, anti stage specific embryonic antigen 1 (SSEA1) (Solter and Knowles 1978), TRA-1-60 and TRA-1-81 (Andrews *et al* 1984a), TRA-2-54, anti 15 liver/kidney/bone, alkaline phosphatase (Andrews *et al* 1984c), TRA-1-85, anti-Ok(a) (Williams *et al* 1988) and anti-Thy1 (McKenzie and Fabre 1981). FITC-labeled goat anti-mouse IgM or anti-IgG was used as the secondary antibody, as appropriate to the isotype of the primary antibody. In some experiments, cells that were either positive or negative for SSEA3 and SSEA1 were isolated by fluorescence activated cell sorting 20 (Andrews *et al* 1987).

20 **RT-PCR Analysis of Gene Expression**

Total RNA was extracted from ES cells, sorted for antigen expression, using Tri Reagent (Sigma) and was treated with DNase (DNA-Free, Ambion). Subsequently, no genomic contamination could be detected by PCR (data not shown). One microgram of total RNA was used for each reverse transcription (RT) reaction as previously described (Duran *et al*, 2001). Equal aliquots of RT product were then subjected to PCR using the primers 25 and conditions summarized in Table 1. In all cases 35 PCR cycles were used except for Oct4 (28 cycles), Rex-1 (30 cycles) and β -actin (20 cycles). The PCR primers for AFP were those reported by Schuldiner *et al* (2000); those for NeuroD1 and β -actin were reported by Duran *et al* (2001). The remaining primers were designed by using the

PRIMERSELECT package from the DNASTAR suite of programs, and the specificity of the RT-PCR products was confirmed by sequencing.

The putative human *Rex1* sequence was obtained by searching for ESTs homologous to mouse *Rex1* (ZFP-42, NM_009556) using the BLAST protocol (Altschul, et al 1990).
5 One EST (AW665472) mapped to the 3' end of an open reading frame predicted by Fgenesh analysis (CGG Web Server at <http://genomic.sanger.ac.uk>) on chromosome 4q35.2 (a region syntenic to mouse *Rex1* on chromosome 8). The protein translation of this ORF shows very high homology (unpublished data) with the mouse Rex-1 protein
10 (Ben-Shushan et al 1998). The complete ORF was isolated by PCR from human ES clone H7 cDNA and sequenced, in triplicate. The human *Rex1* sequence was submitted to Genbank (AF450454).

15

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CLAIMS

- 5 1. A method to manipulate the phenotype of a stem cell comprising;
 - (i) providing a cell which has been transfected with a nucleic acid molecule wherein said molecule includes a promoter which comprises at least one nucleic acid sequence motif which confers substantially stem cell specific expression on at least one selectable marker gene;
 - 10 (ii) providing conditions conducive to the proliferation of said cell in (i) above; and optionally
 - (iii) maintaining and/or storing said cell.
- 15 2. A method according to claim 1 wherein said conditions are *in vitro* cell culture conditions.
3. A method according to claim 1 or 2 wherein said promoter is a synthetic promoter.
4. A method according to any of claims 1-3 wherein said motif binds the transcription factors of the POU domain family.
- 20 5. A method according to claim 4 wherein said motif binds the transcription factor Oct-3/4.
- 25 6. A method according to claim 5 wherein said motif binds an alternatively spliced form of Oct-3/4 selected from the group consisting of; Oct-5 or -6.
7. A method according to any of claims 1-6 wherein said motif is derived from the FGF-4 promoter.
- 30 8. A method according to claim 7 wherein said FGF-4 promoter is of human origin.
9. A method according to any of claims 1-8 wherein said motif is at least one copy of the sequence CTTTGTT and ATGCAAAT.

10. A method according to any of claims 1-8 wherein said motif is at least one copy of the sequence CTTTGTT and ATGCTAAT.
11. A method according to claim 9 wherein said motif comprises at least one copy of the motif CTTTGTTXATGCAAAT wherein X is at least one nucleotide base which serves as a linking molecule.
5
12. A method according to claim 10 wherein said motif comprises at least one copy of the motif CTTTGTTXATGCTAAT wherein X is at least one nucleotide base which serves as a linking molecule.
10
13. A method according to claims 9 or 11 wherein said motif is CTTTGTTcgaATGCAAAT.
15
14. A method according to any of claims 1-13 wherein said nucleic acid molecule comprises at least 2 motifs.
20
15. A method according to any of claims 1-13 wherein said nucleic acid molecule comprises a plurality of motifs.
25
16. A method according to claim 15 wherein said nucleic acid molecule comprises at least 3, 4, 5, 6, 7, 8, 9 or 10 motifs.
30
17. A method according to any of claims 1-6 wherein said motif is derived from the *Rex-1 (zfp-42)* promoter.
25
18. A method according to claim 17 wherein said *Rex-1* promoter is of human origin.
19. A method according to claim 17 wherein said *Rex-1* promoter is of murine origin.
30
20. A method according to any of claims 17-19 wherein said *Rex-1* promoter is ATTTGCAT.

21. A method according to any of claims 1-20 wherein said stem cells are selected from a group consisting: haemopoietic stem cells; neural stem cells; bone stem cells; muscle stem cells; mesenchymal stem cells; trophoblastic stem cells; epithelial stem cells endodermal stem cells; embryonic stem (ES) cells; 5 embryonal germ (EG) cells.

22. A method according to claim 21 wherein said epithelial stem cells are derived from organs selected from the group consisting of; gastrointestinal mucosa, kidney, bladder, mammary glands, uterus, prostate and endocrine glands.

10 23. A method according to claims 21 or 22 wherein said endocrine gland is the pituitary.

24. A method according to claim 21 wherein said endodermal stem cells are derived 15 from organs selected from the group consisting of; liver, pancreas, lung and blood vessels

25. A method according to any of claims 21-24 wherein said stem cells are primate.

20 26. A method according to claim 25 wherein said stem cells are human.

27. A method according to any of claims 21-26 wherein said embryonic stem cells are embryonal carcinoma cells.

25 28. A method according to claim 27 wherein said embryonal carcinoma cells are TERA2 cells.

29. A method according to claim 28 wherein said embryonal carcinoma cells are NTERA 2 cells.

30 30. A method according to any of claims 1-29 wherein said selectable marker is a gene encoding a polypeptide capable of fluorescence emission when excited by light.

31. A method according to claim 30 wherein said selectable marker polypeptide is selected from the group consisting of; BFP, CFP, YFP, GFP, RFP and variants thereof.

5 32. A method according to any of claims 1-29 wherein said selectable marker is a gene encoding a polypeptide which confers resistance to antibiotics selected from the group consisting of; β lactamase, tetracycline resistance polypeptide, chloramphenicol acetyltransferase, aminoglycoside phosphotransferase, hygromycin phosphotransferase; puromycin N acetyltransferase, or variants thereof.

10

15 33. An isolated nucleic acid molecule comprising a promoter of a gene, wherein said promoter comprises a motif, and wherein said motif, has substantially stem cell specific expression and which is operably linked to at least one selectable marker, wherein said motif is bound by a transcription factor of the POU family.

34. An isolated nucleic acid molecule according to claim 33 wherein said transcription factors is Oct-3/4.

20 35. An isolated nucleic acid molecule according to claim 34 wherein said transcription factors is Oct-5 and-6.

36. An isolated nucleic acid molecule according to any of claims 33- 35 wherein said motif is at least one copy of the sequence CTTTGTT and ATGCAAAT.

25

37. An isolated nucleic acid molecule according to any of claims 33-35 wherein said motif is at least one copy of the sequence CTTTGTT and ATGCTAAT.

38. An isolated nucleic acid molecule according to claim 36 wherein said motif comprises at least one copy of the motif CTTTGTTXATGCAAAT wherein X is at least one nucleotide base which serves as a linking molecule.

30

39. An isolated nucleic acid molecule according to claim 37 wherein said motif comprises at least one copy of the motif CTTTGTTXATGCTAAT wherein X is at least one nucleotide base which serves as a linking molecule.

5 40. An isolated nucleic acid molecule according to claim 33 wherein said motif is derived from the *Rex-1 (zfp-42)* promoter.

41. An isolated nucleic acid molecule according to claim 40 wherein said *Rex-1 (zfp-42)* promoter is of human origin.

10 42. An isolated nucleic acid molecule according to claim 40 wherein said *Rex-1 (zfp-42)* promoter is of murine origin.

15 43. An isolated nucleic acid molecule according to any of claims 33-42 wherein said selectable marker encodes a prodrug activating polypeptide.

20 44. An isolated nucleic acid molecule according to claim 43 wherein said prodrug activating polypeptide is selected from the group consisting of: thymidine kinase; triphosphate cytosine deaminase; 5' guanosine -xanthine phosphoribosyl transferase; purine nucleoside phosphorylase; nitroreductase; CYP 2B1 (cytochrome P450); CYP 4B1 (cytochrome P450); Cytochrome P450; Varicella zoster virus thymidine kinase (VZVtk); β -glucosidase; β -lactamase; β -glucuronidase; carboxylesterase; alkaline phosphatase; or carboxypeptidase G2.

25 45. A vector comprising a nucleic acid molecule according to any of claims 33-44.

46. A stem cell transfected with an isolated nucleic acid molecule according to any of claims 33-44 or with a vector according to claim 45.

30

47. A stem cell according to claim 46 wherein said stem cell is selected from a group consisting: haemopoietic stem cell; neural stem cell; bone stem cell; muscle stem cell; mesenchymal stem cell; trophoblastic stem cell; epithelial stem cell; endodermal stem cell; embryonic stem (ES) cell; or embryonal germ (EG) cell.

5 48. A stem cell according to claim 47 wherein said epithelial stem cells are derived from organs selected from the group consisting of; gastrointestinal mucosa, 10 kidney, bladder, mammary glands, uterus, prostate and endocrine glands.

49. A stem cell according to claims 47 or 48 wherein said endocrine gland is the pituitary

15 50. A stem cell according to claim 47 wherein said endodermal stem cells are derived from organs selected from the group consisting of; liver, pancreas, lung and blood vessels.

51. A stem cell according to any of claims 47-50 wherein said stem cells are primate.

20 52. A stem cell according to claim 51 wherein said stem cells are human.

53. A stem cell according to claim 47 wherein said embryonic stem cells are embryonal carcinoma cells.

25 54. A stem cell according to claim 53 wherein said embryonal carcinoma cells are TERA2 cells.

55. A stem cell according to claim 54 wherein said embryonal carcinoma cells are 30 NTERA 2 cells.

56. A differentiated cell transfected with a DNA molecule or vector according to any of claims 33-44 or with a vector according to claim 45.

57. A differentiated cell according to claim 56 wherein said differentiated cell is selected from the group consisting of: a nerve cell; a mesenchymal cell; a muscle cell (cardiomyocyte); a liver cell; a kidney cell; a blood cell (eg erythrocyte, CD4+ lymphocyte, CD8+ lymphocyte); pancreatic β cell; epithelial cell (eg lung, 5 gastric,); and a endothelial cell.

58. A cell culture comprising a transfected stem cell according to any of claims 46-55.

59. A cell culture comprising a differentiated cell according to claim 56 or 57.

10

60. An organ/tissue comprising at least one differentiated cell according to claim 56 or 57.

15

61. A method to ablate a differentiated cell which has or is de-differentiating to a stem cell or lineage restricted stem cell comprising exposing said cell to an agent to which said cell has been sensitized.

62. A method according to claims 61 wherein said method is an *in vivo* method.

20

63. A method according to claims 61 wherein said method is an *in vitro* method.

25

64. A method according to any of claims 61-63 wherein said agent is selected from the group consisting of: ganciclovir; 5-fluorouracil (5-FU); 6-Tg triphosphate; 6-methylpurine; hydroxylamine; 4-hydroperoxycyclo-phosphoamide; DNA-alkylating; alkylating metabolites; araM-MP; cyanide; vinca alk; phenolmustard; SN-38; phenolmustard; etoposide; or benzoic acid mustards.

65. A method according to any of claims 61-63 wherein said agent is diphtheria toxin or pseudomonas exotoxin.

30

Figure 1

1/11

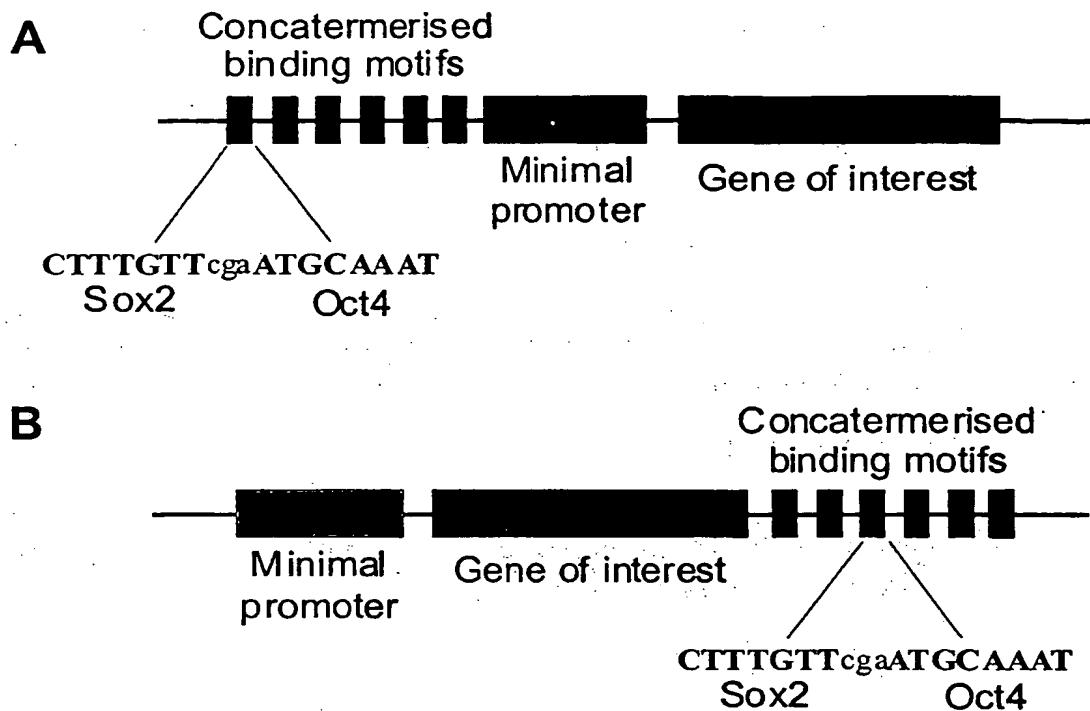


Figure 2Minimal promoter sequences

Mouse FGF4 promoter -64 to +101

cgcggggcaggcgagtaggagggggcgccggctataataccactgctccggagggctggcgccggggactatcc
cgccaccgttgcgtccctattgtctcgctacttaggtctgtgcgcagcactcaccgaactcacggcccgcagctcgaact
cacgcac

mouse FGF4 promoter -64 to +116

cgcggggcaggcgagtaggagggggcgccggctataataccactgctccggagggctggcgccggggactatcc
cgccaccgttgcgtccctattgtctcgctacttaggtctgtgcgcagcactcaccgaactcacggcccgcagctcgaact
cacgcacggcccgccggccgg

Human FGF4 promoter -84 to +101

cgcgggagcgggcgagtaggagggggcgccggctataataccactgctccggagggctggcgccggctggcgctcagg
gaggcgcaactgctccctcagactcccgactcccgccgcgttccgcggctcgccatgcagccgggta
gagcccgccggccggggccccgtcg

Human FGF4 promoter -84 to +237

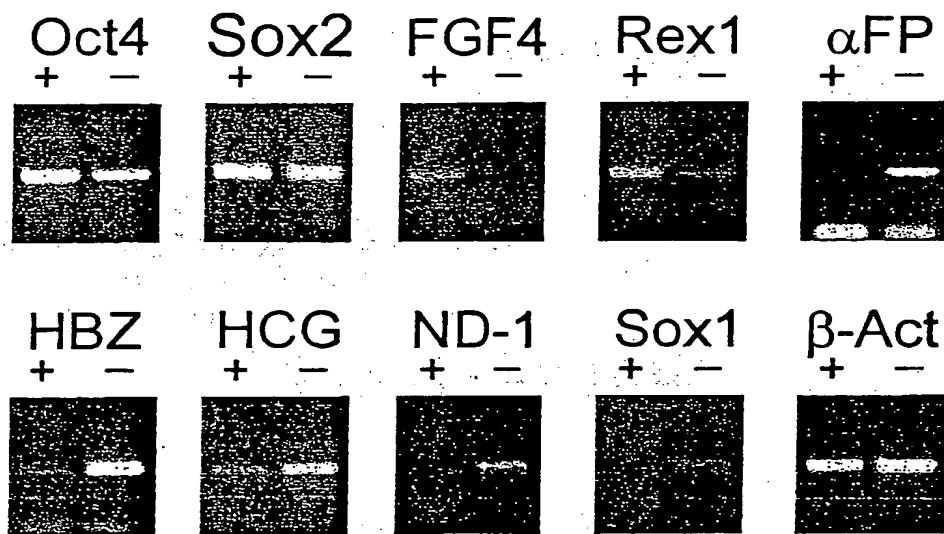
cgcgggagcgggcgagtaggagggggcgccggctataataccactgctccggagggctggcgccggctggcgctcagg
gaggcgcaactgctccctcagactcccgactcccgccgcgttccgcggctcgccatgcagccgggta
gagcccgccggccggggccccgtcgcttgcctccgcacccgtcgactcccgccgaggtcgccgtc
gctcccgccggccacaggcgcaactcccgccgactgaccgcctgaccgacgcacggcc
ctcgccgg

Herpes Simplex Thymidine Kinase minimal promoter

ccccgcccaggctacttgcgaattcgaacacgcagatgcaggcggggcggcgcgggtccagggtccacttcgcatt
attaagggtgacgcgtgtggctcgaataccgagcgaccctgcaggcaccgcgttaacagcgtcaacagcgtccgcaga
tct

Figure 3

SSEA3 Sort



SSEA1 Sort

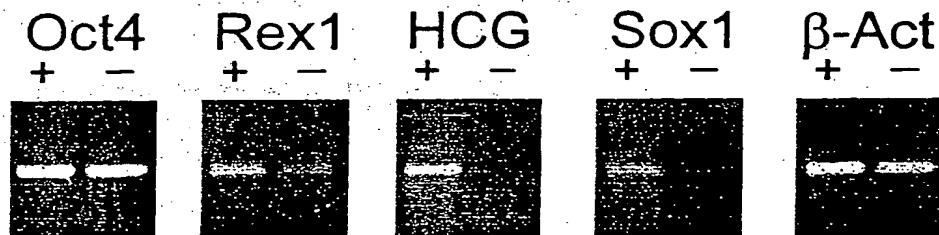


Figure 4

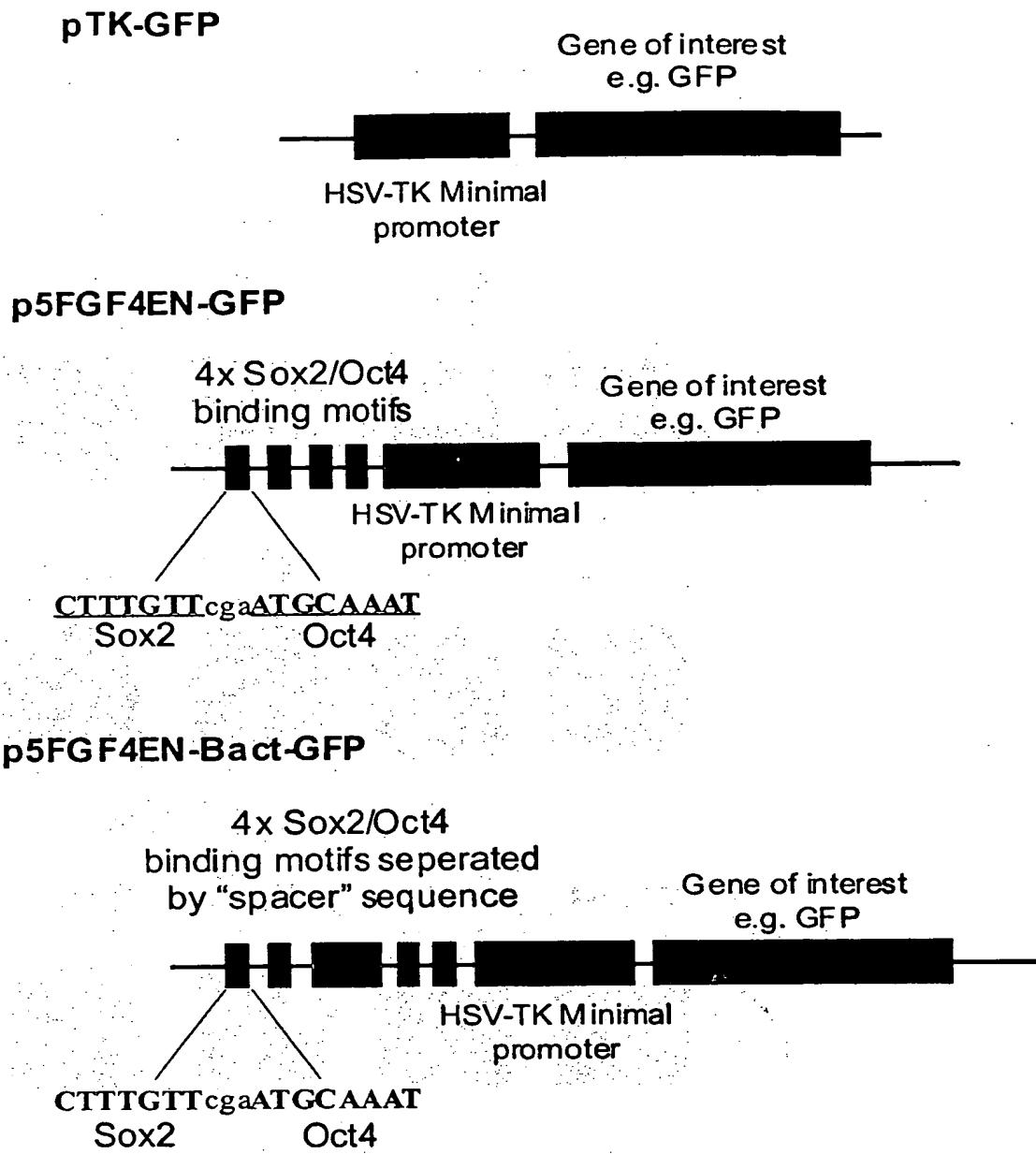


Figure 5

4x human Sox2/Oct4 binding motif

HSV-TK minimal promoter

ccccggccaggcttagtcattggcgaattcaaacacgcagatgcagtcggggcggcggtccagggtccacttcgcattaaaggtaacgcgtgtggcctcaataccgagcgaccctgcagcgaccgcataacagcgtcaacagcgtgcccggcagatctt

pd2EGFP-1 (Clontech)

IRES-Puro

7/11

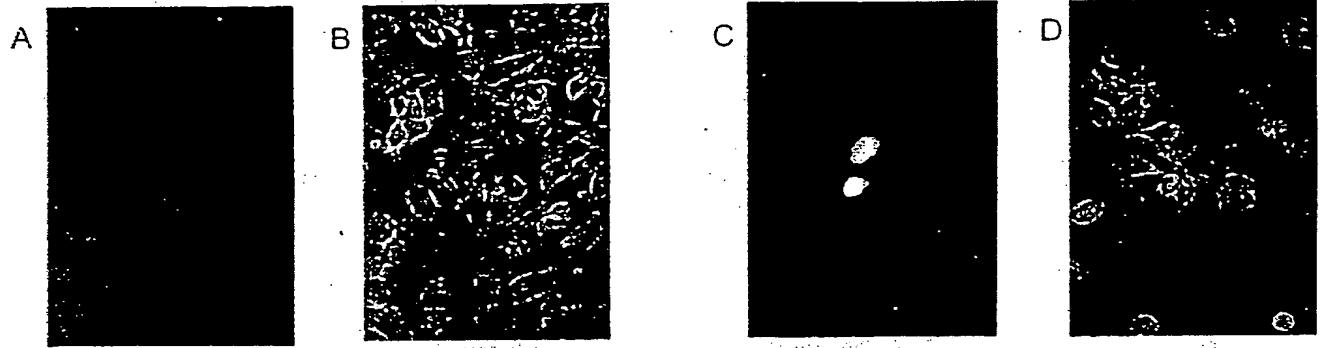
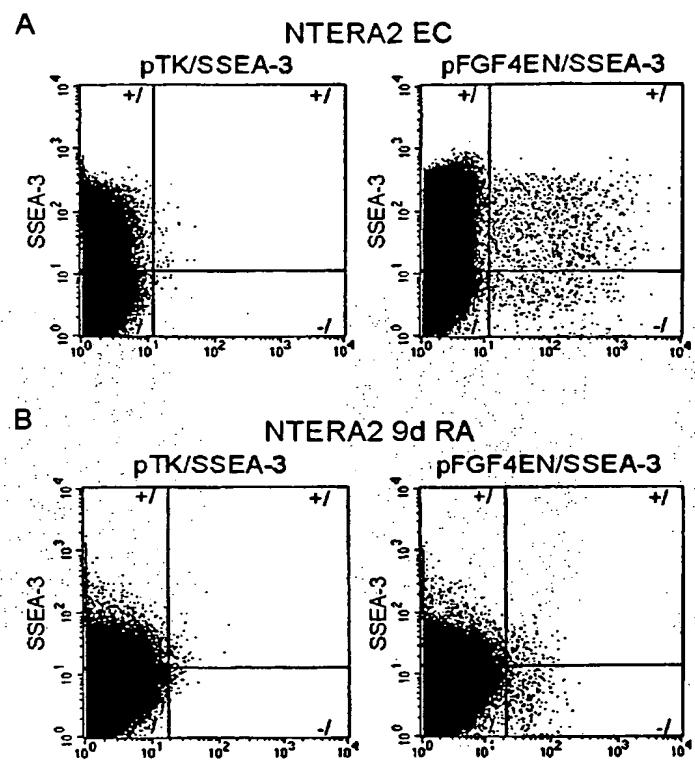


Figure 6

SUBSTITUTE SHEET (RULE 26)

Figure 7

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SUBSTITUTE SHEET (RULE 26)

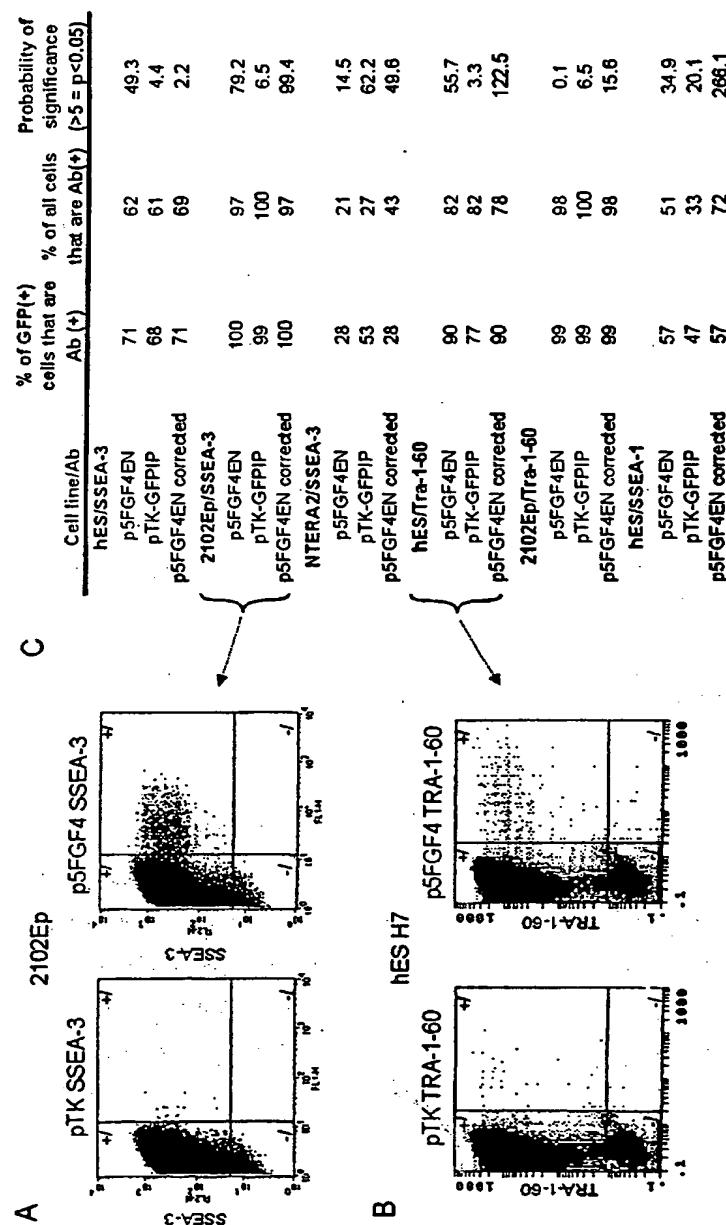


Figure 8 9/11

Table 1

10/11

Gene	Prodrug	Active drug
Thymidine kinase (HSVtk)	Ganciclovir	Ganciclovir
Triphosphate cytosine deaminase (CD)	5-Fluorocytosine (5-FC)	5-Fluorouracil (5-FU)
Guanosine -xanthine phosphoribosyl	6-Thioguanine (6-Tg)	6-Tg triphosphate
Transferase (gpt)		
Purine nucleoside phosphorylase	6-MeP-dR	6-Methylpurine
Nitroreductase (NTR)	CB1954	Hydroxylamine alkylating agents
CYP 2B1 (cytochrome P450)	Cyclophosphamide	4-hydroperoxycyclo- phosphamide
CYP 4B1 (cytochrome P450)	2-Aminoanthracene (2-AA)	DNA-alkylating Agents
Cytochrome P450	4-Ipomeanol	Alkylating metabolites
Varicella zoster virus thymidine	AraM	AraM-MP
Kinase (VZVtk)		
β-glucosidase	Amygdalin	Cyanide
β-lactamase	Vinca cephaloid	Vinca alk
β-glucoronidase	Phenolmustard glucoronide	Phenolmustard
Carboxylesterase	Topoisomerase 1 inhibitors	SN-38
Alkaline phosphatase	Phenolmustard phosphate	Phenolmustard
	Etoposide phosphate	Etoposide
Carboxypeptidase G2	Benzoic acid mustard	Benzoic acid mustards
Glutaminases		

TABLE 2: PCR PRIMERS USED TO DETECT GENE EXPRESSION IN HUMAN ES CELLS

Gene	Accession No.	Sense (5'-3')	Antisense (5'-3')	Product position	Anneal Temp °C	Product size (bp)
Oct4	NM_00270	CGACCCATCTGCCGGCTTGAG	CCCCCTGTCCCCCATTCCTTA	361-937	60	577
Sox2	Z31560	CCCCGGGGCAATAGCA	TGGGGCGGGGAGATACAT	410-857	60	448
Trf4	NM_002007	CTACAAACGCCTACGGAGTCCCTACA	GTTGCCACAGAAAAGTCAGAGTTG	814-1183	55	370
Rex1	AI450454	GCGTACCGAAATTAAAGTCCAGA	CAGCATCCTAAACAGCTCGCAGAAT	793-488	56	306
ATP	NM_001134	AGAACCTGTACAAAGCTGTG	GACAGCAAAGCTGAGGAATGTC	473-1148	57	676
HBZ	MZ4173	CTGACCAAGACTGAGAGGAC	ATGTGTCGATGCTCTTCAC	36-259	61	224
JICG	NM_000737	CAGGGGACCGCACCAAGGATG	GTGGGAGGGATGGGGTGTCC	349-858	62	510
NeuroD1	NM_002500	AAGCCCATGAAACGCCAGAGGAGCT	AGCTGTCCATGGTACCGTAA	240-818	66	579
Sox1	Y13436	CTCACTTCCCTCCGGTTGCTTCC	TGCCCTGGTCTTGTCCCTCATCC	1588-2435	58	848
β-Actin	NM_001101	ATCTGGCACCACTCTACAATG AGCTGCG	CGTCATACTCTGCTGATCCAC ATCTGC	326-1163	60	838

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(54) Title: STEM CELL CULTURE

(57) Abstract: We describe a method to manipulate the phenotype of stem cells, preferably embryonic stem cells (ES), including nucleic acids and vectors used in said methods.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 03/01111

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 C12N15/87 C12N15/85 C12N5/10 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 02/061033 A (YISSUM RES DEV CO) 8 August 2002 (2002-08-08) the whole document	1-60
X	EIGES RACHEL ET AL: "Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells" CURRENT BIOLOGY, vol. 11, no. 7, 3 April 2001 (2001-04-03), pages 514-518, XP001155602 ISSN: 0960-9822 the whole document	1-60

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the International filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the International filing date but later than the priority date claimed

- *T* later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- *&* document member of the same patent family

Date of the actual completion of the International search

15 October 2003

Date of mailing of the international search report

14.01.04

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Giebelter, K

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/GB 03/01111

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BEN-SHUSHAN ET AL: "Rex1, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site" MOLECULAR AND CELLULAR BIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, US, vol. 18, no. 4, April 1998 (1998-04), pages 1866-1878, XP002159568 ISSN: 0270-7306 the whole document	1-60
X	SCHOORLEMMER J ET AL: "OCTAMER-DEPENDENT REGULATION OF THE KFGF GENE IN EMBRYONAL CARCINOMA AND EMBRYONIC STEM CELLS" MECHANISMS OF DEVELOPMENT, ELSEVIER SCIENCE IRELAND LTD, IE, vol. 36, no. 1/2, 1991, pages 75-86, XP000856428 ISSN: 0925-4773 the whole document	1-60
X	MA YAN-GAO ET AL: "Transcriptional regulation of the murine k-FGF gene in embryonic cell lines" DEVELOPMENTAL BIOLOGY, vol. 154, no. 1, 1992, pages 45-54, XP009018601 ISSN: 0012-1606 the whole document	1-60
X	WO 94/24274 A (MOUNTFORD PETER SCOTT ; SMITH AUSTIN GERARD (GB); UNIV EDINBURGH (G) 27 October 1994 (1994-10-27) the whole document	1-3, 14-16, 21-32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 03/01111

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1 and 3-32 when relating to in vivo uses encompass methods of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see PCT/ISA/210 annex

Remark on Protest

The additional search fees were accompanied by the applicant's protest

No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-60

Method to manipulate the phenotype of a stem cell comprising
(i) providing a cell which has been transfected with a nucleic acid molecule wherein said molecule includes a promoter which comprises at least one nucleic acid sequence motif which confers substantially stem cell specific expression on at least one selectable marker gene;
(ii) providing conditions conductive to the proliferation of said cell in (i) above; and optionally
(iii) maintaining and/or storing said cell;

An isolated nucleic acid molecule comprising a promoter of a gene, wherein said promoter comprises a motif, and wherein said motif, has substantially stem cell specific expression and which is operatively linked to at least one selectable marker, wherein said motif is bound by a transcription factor of the POU family;

A vector comprising said nucleic acid molecule; Cell (culture)/ organ/ tissue containing said nucleic acid molecule or vector.

2. claims: 61-65

Method to ablate a differentiated cell which has or is de-differentiating to a stem cell or lineage restricted stem cell comprising exposing said cell to an agent to which it has been sensitized.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 03/01111

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